REMARKS

This amendment is being filed in response to the final Office Action mailed December 18, 2003, and in connection with the accompanying Request for Continued Examination. Claims 1 to 70 are pending. Claims 1 to 32 and 56 to 70 stand withdrawn from consideration as directed to non-elected subject matter. Accordingly, claims 31 to 55 are under consideration.

Regarding the Information Disclosure Statements

Applicants submit herewith an Information Disclosure Statement (IDS) that includes references previously filed with IDS', and accompanying references. Applicants respectfully request that the Examiner consider the references filed with the IDS, and initialize the left column of form PTO-1449 indicating that each of the references have been considered, returning a copy to the undersigned.

REJECTIONS UNDER 35 U.S.C. §112

The rejection of claims 43 and 44 under 35 U.S.C. §112, first paragraph, as allegedly lacking an adequate written description, is respectfully traversed. Allegedly, "the evidence of record has not described which variants or subsequences of gut endocrine promoters possess the biological activity of a gut endocrine promoter." [Office Action, page 4]

The specification adequately describes claims 43 and 44. Applicants respectfully point out that in order to satisfy the written description requirement under 35 U.S.C. §112, first paragraph, the specification need only apprise the skilled artisan of the invention in sufficient detail to demonstrate Applicants had possession of the invention. Possession may be shown by "any description of sufficient, relevant, identifying characteristics so long as a person skilled in the art would recognize that the inventor had possession of the claimed invention." *Purdue Pharma L.P. v. Faulding Inc.*, 230 F.3d 1320, 1323 (Fed. Cir. 2000).

The Federal Circuit explained that a description may be achieved by means of a recitation of structural features common to the members of the genus, which constitute a substantial portion of the genus. *Reagents of the Univ. Calif. v. Eli Lilly* 119 F.3d 1559, 1568 (Fed. Cir. 1997) For biological molecules, identifying characteristics can include, *inter alia*, sequence, structure and length. An adequate written description does not require the disclosure of every

species encompassed by the claims, even in an unpredictable art. *In re Angstadt*, 537 F.2d 498, 502-503 (CCPA 1976). In *Lilly*, the court explained that "every species in a genus need not be described in order that a genus meet the written description requirement." *Id.* (*citing Utter v. Hiraga*, 845 F.2d 993, 998-99 (Fed. Cir. 1988)). Thus, a description of every gut endocrine promoter, or gut endocrine promoter variant or subsequence, is not necessary in order to satisfy the written description requirement under 35 U.S.C. §112, first paragraph for claims 43 and 44.

Here, in view of the specification and knowledge in the art, which discloses relevant, identifying characteristics of gut endocrine promoters and enhancers, the elements that confer and are likely to confer activity, one skilled in the art would be apprised of gut endocrine promoter and enhancer variants and subsequences. Given that one skilled in the art would know identifying characteristics of various gut endocrine promoter, the skilled artisan would also know gut endocrine promoter and enhancer sequences that could be mutated or deleted without destroying activity. Consequently, because the skilled artisan would know sequences of gut endocrine promoters and enhancers that could tolerate substitutions or deletions without destroying activity, the skilled artisan would know of numerous functional gut endocrine promoter and enhancer variants and subsequences.

As previously pointed out in the record, the specification discloses various gut endocrine promoters. Specific non-limiting examples include GIP promoter, secretin promoter, gastrin promoter, cholecystokinin (CCK) promoter, proglucagon promoter, chromogranin A promoter and chromogranin B promoter.

For the GIP promoter, several transcriptional control elements, including two TATA boxes and two CCAAT-like boxes are present. The specification also discloses potential AP-1 and AP-2 sites, as well as a cAMP response element, a potential insulin-response element located upstream of the putative transcription start site and two GATA binding motifs (page 13, lines 10-16). The specification additionally discloses that mutations in the distal and proximal GAT motifs reduced GIP promoter activity 90% and 35%, respectively (page 13, lines 17-19). The specification discloses that a GIP promoter containing one or more of these variants or subsequences is an example of a sequence that can retain glucose-regulatable or tissue specific (gut) expression of an operably linked nucleic acid. (page 13, lines 21-26). Thus, the skilled artisan would know sequence regions of GIP promoter that could be varied or deleted and still confer at last partial activity.

To illustrate, TATA boxes or CCAAT boxes appear frequently in promoters, and are known to be important for promoter activity. The skilled artisan would therefore know that deleting or mutating the TATA boxes or CCAAT boxes would reduce GIP promoter activity. Consequently, the skilled artisan would also know that nucleotides outside of TATA boxes or CCAAT boxes could be mutated or deleted without destroying promoter function and, therefore, would know of GIP promoter variants and subsequences having function.

Other investigators have characterized GIP variants and subsequences having activity. For example, the sequence region between –180 and +14 of human GIP promoter conferred basal promoter activity in an insulinoma cell line (Someya et al., 1993 FEBS Lett., 317:67). The first 193bp upstream of the transcription initiation site of the rat GIP promoter was able to direct approximately the same level of expression as a 943bp promoter fragment in STC-1 cells (Boylan et al., 1997 J Biol Chem, 272:17438). Thus, the skilled artisan would have known that a sequence from about -180 and +14 or about the first 193bp upstream of the transcription initiation site would have at least partial GIP promoter activity and, as such, would be a functional subsequence of gut endocrine promoter of claims 43 and 44.

Furthermore, DNase I footprinting and gel mobility shift assays identified one near-canonical and one atypical cAMP response elements (CRE) at positions –351 (AGACGTGA) and –158 (TCACCAAT), respectively. Both CRE sites are essential for basal promoter activity (Someya et al., 1993 FEBS Lett., 317:67). Thus, the skilled artisan would have known that the CRE's are important for GIP promoter function and, as such, that nucleotides outside of the CRE's could be mutated or deleted without destroying promoter activity.

As to gut promoters and enhancers other than GIP promoter, although promoter sequences may be different from each other, given the knowledge in the art regarding the sequence elements that confer and are likely to confer activity in a given promoter, the skilled artisan would know which sequences, if varied or deleted, are likely to reduce function of a given promoter or enhancer. Again, analogous to GIP promoter, because one skilled in the art would have known sequences that have and are likely to have a role in activity of a given gut endocrine promoter or enhancer, one skilled in the art would also know variants and subsequences of the gut endocrine promoter or enhancer that would retain at least partial activity.

For secretin, promoter sequences between 174 and 53 bp upstream from the secretin transcriptional start site confer maximal expression (Wheeler et al., 1992 Mol Cell Biol,

12:3531). Within this region there are four cis-acting elements characterized in transient expression assays (Mutoh et al., 2000 Aliment Pharmacol Ther, 14 Suppl 1:170; Nishitani et al., 1995 J Clin Gastroenterol, 21 Suppl 1:S50). Thus, the skilled artisan would have known that the 174 and 53 bp region is sufficient to confer activity and, furthermore, that deleting the four cis acting elements within this region would likely destroy secretin promoter activity. The BETA2 protein binds to the E box in the secretin gene and interacts with p300 to activate secretin transcription (Mutoh et al., 1998 Genes Dev, 12:820). A 1.6kb 5' flanking sequence of rat secretin gene conferred tissue-specific, developmentally regulated expression in mice (Lopez et al., 1995 J Biol Chem, 270:885).

For gastrin, transcription was shown to be increased by epidermal growth factor (Godley and Brand, 1989 Proc Natl Acad Sci USA, 86:3036). An EGF response element (gERE) is located between -54 to -68 bp 5' of the transcription initiation site of human gastrin promoter (Merchant et al., 1991 Mol Cell Biol, 11:2686). Gastrin transcription in islet cells is activated by a cis-regulatory sequence containing a RAP1-like binding site (Simon et al., 1994 FEBS Lett, 351:340). Sp transcription factor members Sp1 and Sp3, which have previously been identified as important for activating a number of promoters via cis-acting elements, bind to this RAP-1 site (Simon et al., 1997 FEBS Lett, 411:383). A positive cis-regulatory element (CACC) is located from position -109 to -100 bp (Tillotson et al., 1994 J Biol Chem, 269:2234-2240). RIN ZF, a member of the Cys2-His2 zinc finger family, binds to this CACC element and regulates gastrin gene expression (Tillotson et al., 1994 J Biol Chem, 269:2234). Thus, in view of the foregoing, the skilled artisan would have known of a variety of sequence elements within gastrin promoter that have and are likely to have a role in activity and, as such, would also have known of a variety of gastrin promoter variants and subsequences that retain at least partial activity.

Cholecystokinin (CCK) gene region from –100 to –20 relative to the transcriptional start site contains an E-box element (-97 to –92 CANNTG), a combined CRE/TRE element (-79 to –73), a GC-rich box (-39 to –32) and a TATA-box. Since each of these elements are known by the skilled artisan to be important in activity of many other promoters, the skilled artisan would have known elements within CCK promoter that have and are likely to have a role in activity and, as such, would also have known of a variety of CCK promoter variants and subsequences that retain at least partial activity.

The proglucagon gene spans approximately 10kb (Heinrich et al., 1984 J Biol Chem, 259:14082; White and Saunders, 1986 Nucleic Acids Res, 14:4719) and 300 base pairs of the 5'-flanking region of rat proglucagon gene conferred specific expression in islet cell lines. Three transcriptional control elements, G2 (-181 to –202) and G3 (-265 to –286), each displayed enhancer activity in alpha cells (Philippe et al., 1988 Mol Cell Biol, 8:4877). The third control element, G1 proximal promoter element (-52 to –100), exhibited low intrinsic transcriptional activity but was important for specific expression in alpha cells. A composite control element, G4, located upstream of G1 between nucleotides -100 and –140, functions as an islet-specific activator in both glucagon- and insulin-producing cells and is inactive in nonislet cells (Cordier-Bussat et al., 1995 Mol Cell Biol, 15:3904). A cyclic-AMP responsive element (CRE) is also present at positions -291 to –298 (Knepel et al., 1990 Mol Cell Biol, 10:6799). Thus, in view of the foregoing, the skilled artisan would have known of a variety of sequence elements within proglucagon promoter that have a role in activity and, as such, would also have known of a variety of proglucagon promoter variants and subsequences that retain at least partial activity.

Chromogranin A gene has a glucocorticoid response element located at position -583 to -597 bp, which confers glucocorticoid regulation (Rozansky et al., 1994 J Clin Invest, 94:2357). An Sp1/Egr-1 site spans -88 to -77 base pairs (bp) and a cyclic AMP-responsive element (CRE) is located at -71 to -64 bp; both are important for gastrin-dependent chromogranin A activation (Hocker et al., 1998 J Biol Chem, 273:34000-34007). Gastrin regulates Sp1 binding to the chromogranin A -88 to -77 bp promoter element, as well as CREB binding to the chromogranin A consensus motif at -71 to -64 bp. Thus, in view of the foregoing, the skilled artisan would have known of a variety of sequence elements within chromogranin A promoter that have and are likely to have a role in promoter activity and, as such, would also have known of a variety of chromogranin A promoter variants and subsequences that retain at least partial activity.

Chromogranin B gene promoter is GC-rich and contains a CATAA motif, a cAMP-responsive element and an Sp1 binding site (Pohl et al., 1990 FEBS Lett, 262:219). Neuroendocrine cell type-specific expression activity is conferred by proximal chromogranin B promoter, from -216 to -91 bp (Mahapatra et al., 2000 Endocrinology, 141:3668), which contains an E box (at -206 bp-201 bp), four G/C-rich regions (at -196 bp-191 bp, -134 bp -127 bp, -125 bp -117 bp, and -115 bp -110 bp), and a cAMP response element (CRE; at -102 bp -95 bp). Thus, in view of the foregoing, the skilled artisan would have known of a variety of sequence elements

within chromogranin B promoter that have a role in activity and, as such, would also have known of a variety of chromogranin B promoter variants and subsequences that retain at least partial activity.

In sum, because the skilled artisan would have known sequences that have and are likely to have a role in activity of a wide variety of gut endocrine promoters and enhancers, the skilled artisan would be apprised of sufficient relevant, identifying characteristics of gut endocrine promoters and enhancers. In view of the fact that the skilled artisan would have known these elements in gut endocrine promoters and enhancers, the skilled artisan would also know nucleotides that could be substituted or deleted without destroying gut endocrine promoter or enhancer activity and, therefore, gut endocrine promoter and enhancer variants and subsequences having at least partial activity. Consequently, as gut endocrine promoters and enhancers having variations or deletions that retain activity would be known to one skilled in the art, an adequate written description for claims 43 and 44 is provided. Accordingly, Applicants respectfully request that the rejection under 35 U.S.C. §112, first paragraph, as allegedly lacking an adequate written description, be withdrawn.

The rejection of claims 31 to 55 under 35 U.S.C. §112, first paragraph, as allegedly lacking enablement is respectfully traversed. The Examiner has maintained the rejection.

Claims 31 to 55 are adequately enabled. Here, the grounds for rejection relate solely to an alleged "unpredictability of the gene therapy art." [Office Action, page 5]

Applicants again respectfully point out that this rejection is improper since gene therapy is not required in order to practice claims 31 to 55. Here, claims 31 to 55 do not recite a step of transforming mucosal cells but, rather, "contacting mucosal tissue cells in the subject transformed with a polynucleotide comprising an expression control element in operable linkage with a nucleic acid encoding the therapeutic protein with a nutrient that induces production of the protein in an amount effective to treat the disorder." Consequently, as claims 31 to 55 do NOT require gene therapy to practice the full scope of the invention, Applicants need not enable gene therapy in order to satisfy the enablement requirement under 35 U.S.C. §112, first paragraph. As such, the grounds for rejection of claims 31 to 55 relating to enabling gene therapy is improper and must be withdrawn.

Notwithstanding the foregoing, evidence that mucosal cell transformation and therapeutic levels of protein expression *in vivo* would not require undue experimentation was previously submitted with Applicants' Response. In particular, Applicants previously submitted Exhibit A, a sworn Declaration under 37 C.F.R. §1.132 and accompanying Figures 1-11, executed by Dr. Timothy Kieffer. Exhibit A provided animal studies in which a therapeutic protein (leptin) from translplanted gut cells (GTC-1) was produced in an amount effective to treat obesity and normalize glucose levels in animals. The data in previously submitted Exhibit A therefore corroborates that a disorder can be treated by contacting transformed mucosal tissue cells in a subject with a nutrient that induces production of a protein in an amount effective to treat the disorder, as in claims 31 to 55.

To corroborate that *in vivo* transformed mucosal cells can express relevant levels of protein without undue experimentation, submitted herewith as Exhibit 1, is a sworn Declaration under 37 C.F.R. §1.132 executed by Dr. Anthony Cheung, one of the inventors of the application, and Exhibit 2, accompanying Figures 1-4. Exhibits 1 and 2 include data indicating *in vivo* gene transfer of mucosal tissue with three different transferred genes and expression, using materials and methods disclosed in the specification or known in the art at the time of the invention; see, for example, page 12, lines 17-20; page 37, line 28, to page 38, line 6; page 20, lines 13-14; page 21, lines 7-11; page 12, lines 19-24; page 16, lines 11-12; and page 15, Table 1. Exhibits 1 and 2 also include data indicating that levels of protein encoded by *in vivo* transferred insulin gene would be sufficient to reduce glucose (paragraph 11).

In brief, two different delivery vectors, feline immunodeficiency virus (FIV) and adeno-associated virus (AAV-2), were used to transfer three genes, namely human insulin, red fluorescent protein (DsRed) and green fluorescent protein (GFP), into mucosal tissue of rats (Exhibit 1, paragraph 6). FIV was used to deliver insulin or DsRed into mucosal tissue of animals and AAV-2 was used to deliver GFP into mucosal tissue of animals. Viral vectors were delivered to animals by direct injection into the intestinal wall or by luminal incubation (Exhibit 1, paragraph 9). To detect the transferred DsRed or GFP genes, duodenal mucosa genomic DNA was analyzed for gene copy numbers, and compared to duodenal mucosa genomic DNA of untreated animals (Exhibit 1, paragraph 10). Human insulin was detected by measuring C-peptide levels in blood samples. C-peptide is a by-product of insulin synthesis and is released from insulin-producing cells in an equimolar amount as insulin (Exhibit 1, paragraph 11).

GFP was present in the small intestine following gene transfer by both injection and incubation (Exhibit 1, paragraph 12; and Exhibit 2, Fig. 1). GFP delivered to the duodenum persisted for 2 weeks indicating that the GFP gene integrated into the genomes of intestinal precursor or stem cells of the animals (Exhibit 1, paragraph 12; and Exhibit 2, Fig. 1B).

DsRed was present in the small intestine following gene transfer by both injection and incubation (Exhibit 1, paragraph 13; and Exhibit 2, Fig. 2). DsRed persisted in the duodenum for at least 2 weeks indicating that the DsRed gene integrated into the genomes of intestinal precursor or stem cells of the animals (Exhibit 1, paragraph 13; and Exhibit 2, Fig. 2A). FIV vectors delivered via luminal incubation transferred DsRed into mucosal cells (Exhibit 1, paragraph 13; and Exhibit 2, Fig. 2B).

C-peptide was present in blood following gene transfer into duodenum (24.9±10.7 pM at day 10), whereas prior to human insulin gene transfer, C-peptide was barely detectable (0.7±0.4 pM) (Exhibit 1, paragraph 14; and Exhibit 2, Fig. 3). Plasma human C-peptide levels increased to and remained relatively stable throughout the study (Exhibit 1, paragraph 14; and Exhibit 2, Fig. 3). C-peptide levels reflect insulin levels that would provide a reduction in glucose (Exhibit 1, paragraph 14).

Human insulin expression in the rats was confirmed by analyzing a duodenum sample (collected approximately 128 days after vector injection) for human insulin protein (Exhibit 1, paragraph 15). The data indicate long term expression of human insulin protein in duodenum cells (Exhibit 1, paragraph 15; and Exhibit 2, Fig. 4).

In view of the foregoing *in vivo* data, particularly that two different virus vectors were able to introduce three different genes into mucosal tissue, that at least one of the introduced genes expressed protein at relevant levels in the circulation, and that expressed protein was present in mucosal tissue, Dr. Cheung has concluded that transfer of a gene encoding a therapeutic protein into mucosal tissue cells *in vivo* would not require undue experimentation (Exhibit 1, paragraph 16).

In sum, as claims 31 to 55 do not recite a step of transforming mucosal cells, the claims do NOT require gene therapy. As such, Applicants need not enable gene therapy in order to practice the full scope of claims 31 to 55 or satisfy the enablement requirement under 35 U.S.C. §112, first paragraph. Notwithstanding the foregoing, in view of the data in previously submitted Exhibit A, affirming that therapeutic levels of leptin can be expressed in animal

mucosal cells transformed with a nucleic acid encoding leptin, and further in view of the foregoing data submitted herewith in Exhibits 1 and 2, affirming mucosal cell transformation with different vectors delivering different genes to animals, and expressing therapeutically relevant levels of protein in the animals, claims 31 to 55 are adequately enabled. Accordingly, the rejection under 35 U.S.C. §112, first paragraph, as allegedly lacking enablement, is improper and must be withdrawn.

CONCLUSION

In summary, for the reasons set forth herein, Applicants maintain that claims 31 to 55 clearly and patentably define the invention, respectfully request that the Examiner reconsider the various grounds set forth in the Office Action, and respectfully request the allowance of the claims which are now pending.

If the Examiner would like to discuss any of the issues raised in the Office Action, Applicant's representative can be reached at (858) 509-4065.

Please charge any additional fees, or make any credits, to Deposit Account No. 03-3975.

Respectfully submitted,

Date: 6.16.04

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	Kieffer et al.	Art Unit	:	1632
Serial No.	:	09/804,409	Examiner	:	Paras Jr., Peter
Filed	:	03/12/2001			
Title	:	COMPOSITIONS AND METHODS FOR REGULATED PROTEIN EXPRESSION IN GUT			

Assistant Commissioner of Patents
Washington, DC 20231

DECLARATION OF DR. ANTHONY T. CHEUNG
UNDER 37 C.F.R. §1.132

Sir:

1. I, ANTHONY T. CHEUNG, Ph.D., declare and say I am a resident of Vancouver, British Columbia, Canada. My residence address is: 2507-1331 West Georgia St., Vancouver, BC, Canada V6E 4P1. I received Bachelor of Science degree in Biochemistry from the University of British Columbia in 1993. I received a Doctor of Philosophy degree in Physiology from the Tulane University in 1999. I am currently Chief Scientific Officer of enGene, Inc., in Vancouver, BC, Canada. My curriculum vitae is attached, which reflects my expertise in the areas of molecular biology and biochemistry.
2. I am an inventor of the subject matter claimed in United States Patent Application Serial No. 09/804,409, filed March 12, 2001.
3. I have reviewed the claims that are presently under examination.
4. I understand that claims 31 to 55 have been rejected due to an alleged lack of enablement for *in vivo* gene therapy.
5. I submit this declaration and the studies herein in support of the fact that *in vivo* gene transfer to a mucosal tissue (e.g., intestine) and expression of the encoded protein can be achieved without undue experimentation. I also submit this declaration and the studies herein as evidence that relevant levels of encoded protein in the circulation can be achieved by *in vivo* gene transfer to a mucosal tissue.

Exhibit 1

6. Two different viral vectors were used for gene transfer, a recombinant feline immunodeficiency virus (FIV) and an adeno-associated virus (AAV2). Two different FIV vectors were constructed, one FIV vector that expressed a gene encoding red fluorescent protein driven by a CMV promoter (DsRed; FIV-CMV/DsRed), and a second FIV vector that expressed a gene encoding human insulin driven by a chromagranin A promoter (FIV-cgA/Ins); chromagranin A promoter is a neuroendocrine cell specific promoter. A single AAV2 vector expressing a gene encoding green fluorescent protein (GFP) driven by a CMV promoter was produced (AAV-CMV/hrGFP). Lentivirus and AAV vectors are disclosed in the specification, for example, at page 12, lines 17-20; and at page 37, line 28, to page 38, line 6. Therapeutic and detectable proteins, including insulin and GFP, are disclosed in the specification, for example, at page 20, lines 13-14; and at page 21, lines 7-11, respectively. The specification also discloses that expression control elements such as promoters, can be constitutive (for example, at page 12, lines 19-24; and at page 16, lines 11-12). Chromagranin A promoter for targeting expression of proteins to endocrine cells is disclosed at page 15, Table 1.

7. To obtain the two recombinant FIV vectors packaged into virus particles, 293T cells were co-transfected by a triple-plasmid calcium phosphate transfection protocol. The three plasmids used were: 1) FIV-CMV/DsRed or FIV-cgA/Ins; 2) FIV packaging plasmid expressing FIV gag and pol genes; and 3) envelope plasmid for expressing the VSV-G envelope glycoprotein. Briefly, cells ($1.5x\ 10^7$) were transfected with packaging plasmid (8 μg), envelope plasmid (4 μg) and vector plasmid (8 μg) in 150 mm cell culture dish. Eight hours after transfection, cells were fed fresh medium (DMEM with 10% FBS) and incubated at 37°C overnight. The culture medium was replaced the next morning and the transfected cells transferred to a 32°C incubator. Virus particles were harvested from the culture medium at 48 h and 72 h post-transfection by centrifuging the medium (50,000g for 2 hours), and stored in TNE buffer (50 mM Tris, 130 mM NaCl, and 1mM EDTA) at -80°C until use. FIV titers were determined by real-time quantitative PCR (RQ-PCR) using primers and fluorescent probes specific for DsRed or insulin. A9L mouse

fibroblast cells ($5x10^5$) were infected with purified recombinant FIV (5 μl) and the genomic DNA of infected cells was isolated 48 hours later using Qiagen DNAeasy Tissue Kit (Qiagen, Chatsworth, CA).

8. To obtain the AAV2 vectors packaged into virus particles, an Ad-free, three-plasmid co-transfection method was used. Briefly, 293T cells ($1.5x10^7$) were transfected with CMV/hrGFP plasmid, packaging plasmid and a mini-Ad helper plasmid (10 μg each) as previously described. Eight hours after transfection, growth medium was replaced with (DMEM with 10% FCS). At 72 h post-transfection, cells were harvested by scraping and low speed (1500g) centrifugation. The cell pellet was resuspended in medium (100 ml) and after 3 freeze/thaw cycles in a dry ice/ethanol bath, Benzonase was added (50 units/ml) and incubated for 30 min at 37° C. Cell debris was removed by centrifugation (5000g for 20 min) and the AAV2 particles in the supernatant purified using heparin affinity chromatography. AAV2 vector titers were determined as previously described except virus stock (10 μl) was first incubated with DNase I (10 units, 37°C for 1 hour), followed by Proteinase K digestion (20 μg, 55°C for 2 hours); proteinase K was subsequently inactivated (95°C for 10 min) and AAV DNA was subjected to RQ-PCR.

9. For *in vivo* gene transfer studies, male C57BL/6 mice and Wistar rats were fasted overnight before viral vector delivery. Briefly, animals were anesthetized (isofluorane inhalation) an abdominal incision was made and the duodenum displaced from the abdominal cavity with a glass hook. Virus solution was delivered either by injection to the intestinal wall or by luminal incubation. For injection, the displaced section of the duodenum was elevated from the abdominal cavity and temporarily inflated by injecting saline in the superior direction towards the pyloric sphincter, and the virus injected directly into the duodenal wall at multiple sites using a needle syringe (30G). For AAV-CMV/hrGFP, 120 μl virus (~$1x10^{11}$ genome copies/ml) was injected at 5 separate sites. For FIV-CMV/DsRed, 500 μl virus (~$1x10^9$ infectious unit/ml) was injected at five separate sites. For FIV-cgA/hIns, 500 μl virus (10^8 infectious unit/ animal) was injected at five separate sites. Afterwards, the duodenum was returned to the abdominal cavity

and the incision closed. For luminal incubation, a section of the duodenum (~2 cm) was lifted from the abdominal cavity with a glass hook, a suture tourniquet (5-0) was place around the pyloric sphincter and the isolated section of the duodenum washed with warm saline, followed by incubation with 0.2% DDM (10 min, ~0.12 ml) to minimize the mucous barrier (see the specification, for example, at page 41, line 26, to page 42, line 12). The DDM was removed with warm saline, the isolated section of the duodenum was maintained in an elevated position, and the virus solution injected into the lumen of the duodenum and allowed to incubate in the elevated section for 1 hour. For both AAV-CMV/hrGFP and FIV-CMV/DsRed, 120 μl virus was incubated in the duodenum. Afterwards, the tourniquet was released, the duodenum returned to the abdominal cavity and the incision closed.

10. To detect the AAV transferred DsRed or GFP genes, duodenal mucosa was analyzed. Briefly, mice were sacrificed at 2 days or 14 days after virus delivery, the first 2 cm of the duodenum was dissected free of messentary, opened along its longitudinal axis, and the mucosal surface was washed with ice-cold PBS. The mucosal layer duodenum was gently scrapped from the serosal and muscle layer with a glass slide and snapped frozen in liquid nitrogen. Genomic DNA was isolated from the collected mucosa using the DNAeasy Tissue kit. RQPCR was performed on genomic DNA (1 μg) using the ABI Prism 7000 Sequence Detection System (Applied Biosystem, Foster City, CA). Primers and fluorescent probes specific for DsRed or GFP were used to quantify the number of gene copies in the mucosal genomic DNA. Negative controls (DNA from duodenal mucosa of untreated animals) were included in the assay.

11. To detect insulin after FIV-mediated gene transfer, C-peptide levels were measured in the animals. C-peptide is a by-product of insulin synthesis and is released from insulin-producing cells in an equimolar amount as insulin. Briefly, blood samples were collected from the tail vein before and at 3, 10 and 14 days post-virus vector delivery. The plasma isolated by centrifugation and human C-peptide measured using an ELISA kit, according

to the manufacturer's instructions (Alpco, Windham, NH). The antibody in the assay specifically recognizes human C-peptide and does not cross react with rat C-peptide.

12. GFP was detected following AAV-mediated gene transfer by both injection into and duodenal mucosa incubation (Fig. 1). Thus, AAV vectors deliver genes to the intestine by either direct injection or luminal incubation. The reduction in GFP copy number in the mucosa between the second (Fig. 1A) and fourteenth (Fig. 1B) day is likely due to rapid turnover of duodenal epithelial cells, which have a 2 to 3 day lifespan in mice. Nevertheless, GFP delivered to the duodenum persisted for 2 weeks (Fig. 1B) indicating that the AAV vector successfully integrated GFP into the genomes of intestinal precursor or stem cells of the animals.

13. DsRed was detected in the small intestine following FIV-mediated gene transfer by both injection into and duodenal mucosa incubation (Fig. 2). Injection of FIV vectors into the intestinal wall transferred genes to mucosal cells of the duodenum. DsRed persisted in the duodenum for at least 2 weeks indicating that gut precursor or stem cells were transduced by the FIV vector (Fig. 2A). FIV vectors delivered via luminal incubation transferred DsRed into mucosal cells of the duodenum (Fig. 2B), similar to AAV2.

14. C-peptide was detected in the blood following FIV-mediated gene transfer by injection into duodenum (Fig. 3). Blood levels of human C-peptide were monitored periodically. Before administration, human C-peptide was barely detectable (0.7±0.4 pM) in all animals. After delivery of FIV-cgA/hIns (n=10), plasma human C-peptide levels increased to 24.9±10.7 pM at day 10 and remained relatively stable throughout the study (Fig. 3). These C-peptide levels reflect insulin levels that would provide a reduction in glucose.

15. To confirm human insulin expression in the duodenum of rats receiving the human insulin gene bearing FIV vector, a duodenum sample was collected on ~ day 128 after injection of vectors to the intestinal wall and processed for detection of human insulin protein by immunohistochemistry. In brief, animals were sacrificed and the proximal 2 cm of duodenum collected and fixed in 4% paraformaldehyde for 2 hours. Collected

tissue was incubated in 70% ethanol overnight and subsequently processed for paraffin embedding. Ten micron embedded sections of the duodenum were mounted on glass slides, de-waxed and rehydrated. After 3% hydrogen peroxide treatment and blocking in DAKO Protein Block, sections were treated with an anti-insulin monoclonal antibody (Santa Cruz Biotech, SC-8033). After washing, slides were treated with a biotinylated goat anti-mouse IgG (Vector Laboratories, Burlingame, CA). Slides were then washed and treated with R.T.U. VECTASTAIN ABC Reagent (Vector Laboratories). Signal was developed with DAB peroxidase substrate solution (Vector Laboratories, SK-4100), and the slides examined under a light microscope. The results shown indicate that long-term expression (~128 day) of human insulin protein was detected in cells in the duodenum (Fig. 4).

16. Based upon the foregoing *in vivo* gene transfer data, particularly that two different virus vectors were able to introduce three different genes into mucosal tissue via direct injection or luminal incubation, that at least one of the introduced genes encoded a protein present in the circulation at relevant levels, and that protein was expressed in mucosal tissue, I conclude that transfer of a gene encoding a therapeutic protein into mucosal tissue cells *in vivo* would not require undue experimentation at the time of the invention.

17. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title XVIII of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

June 9th, 2004
Date

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Education

1993	B.Sc.	Major - Biochemistry, University of British Columbia, Vancouver, BC, Canada
1999	Ph.D.	Major - Physiology, Tulane University School of Medicine, New Orleans, LA, USA

Postdoctoral Training

1999-2001	University of Alberta, Edmonton, Canada

PROFESSIONAL EXPERIENCE

2001-present	President and Chief Executive Officer enGene, Inc., Vancouver, BC, Canada
1999	Co-Founder enGene, Inc.
2002-present	Advisory Board Member Biocompare, Inc. S. San Francisco, CA (www.biocompare.com)
1997-1999	Research Associate Div. of Endocrinology, Dept. of Medicine University of Tennessee, Memphis, TN

AWARDS

2001	Travel Award American Diabetes Association 61st Scientific Sessions Philadelphia, PA
2000	Second Prize, The Next Generation Award BioContact Quebec, Quebec City, QC
2000	Canadian Diabetes Association Postdoctoral Fellowship
1999	Phase 1 Technology Commercialization Program Aberta Heritage Foundation for Medical Research
1999	Fellowship Award Aberta Heritage Foundation for Medical Research
1998	Travel Award Joslin Diabetes Centennial Celebration Harvard Medical School, Cambridge, MA
1996	American Heart Association Graduate Student Fellowship.
1995	Chancellor Scholarship. Tulane University, New Orleans, LA
1994	Juvenile Diabetes Federation International Research Scholarship, University of British Columbia, Vancouver, BC
1994	Outstanding Research Award American Federation for Medical Research, Western Student Medical Research Forum. Monterey, CA
1993	Pharmaceutical Manufacturers Association of Canada / Medical Research Council of Canada Research Scholarship in Medicine
1993	Hoechst-Roussel Student Research Scholarship. Vancouver, BC, Canada
1989	University of British Columbia Entrance Scholarship
1989	Ontario Scholar Award Ottawa, ON, Canada

PROFESSIONAL COMMITTEES SERVED

2000-present	Journal Reviewer Journals: *Endocrinology* and *Regulatory Peptides*
1998-1999	Committee Member Subcommittee on Animal Studies Veterans Administration Medical Center, Memphis, TN
1995-1997	Committee Member Graduate School Student Asssociation Tulane University, New Orleans, LA
1994-1995	Committee Member Western Student Medical Research Committee of the American Federation for Medical Research, USA.

PROFESSIONAL MEMBERSHIPS

American Diabetes Association
American Association for the Advancement of Science
American Physiological Society
American Federation for Medical Research, USA. (1993-1995)

BIBLIOGRAPHY

Peer-Reviewed Publications

1. **Cheung A** & Bryer-Ash M: Modified method for the performance of insulin glucose clamp studies in conscious rats. *J. Pharmacol. Toxicol. Methods,* 31 (4) : 215-220, (1994).
1. Bhanot S, Bryer-Ash M, **Cheung A** & McNeil JH: Bis(maltalato)oxovanadium (IV) attenuates hyperinsulinemia and hypertension in spontaneous hypertensive rats. *Diabetes,* 43 : 857-861, (1994).
1. Bryer-Ash M, **Cheung A** & Pederson RA: Feedback regulation of Glucose-Dependent Insulinotropic Polypeptide (GIP) secretion by insulin in conscious rats. *Regulatory Peptides*, 51:101-109, (1994).
1. Qin X., Siaw E, **Cheung AT**, Walters MR: Altered phosphorylation of a 91-kDa protein in particulate fractions of rat kidney after protracted $1,25(OH)_2$Dihydroxyvitamin D_3 or estrogen treatment. *Archives of Biochemistry and Biophysics*, 348(2): 239-246, (1997).

2. **Cheung AT**, Ree D, Kolls JK, Fuselier J, Coy D and Bryer-Ash M: Inhibition of TNF-a improves peripheral and hepatic insulin sensitivity in obese Zucker rats: *Endocrinology* 139(12): 4928-35, (1998).
3. **Cheung AT**, Kusari J, Jansen D, Bandyopadhyay D, Kusari A and Bryer-Ash M: Marked impairment of Protein Tyrosine Phosphatase 1B (PTP-1B) activity in adipose tissue of obese subjects with and without non-insulin dependent diabetes mellitus (NIDDM). *J Lab Clin Med* 134:115-123 (1999).
4. Kieffer TJ, Lam NT, **Cheung AT**. Insulin production from the b-cell: antagonistic roles of GLP-1 and leptin. *Can. J. Diabetes Care* 24:47-57 (2000).
5. **Cheung AT**, Wang J, Ree D, Kolls JK, Bryer-Ash M: Tumor necrosis factor-alpha induces hepatic insulin resistance in obese Zucker (fa/fa) rats via interaction of leukocyte antigen-related tyrosine phosphatase with focal adhesion kinase. *Diabetes* 49(5):810-9 (2000).
6. **Cheung AT**, Dayanandan B, Lewis JT, Korbutt GS, Rajotte RV, Bryer-Ash M, Boylan MO, Wolfe MM, Kieffer TJ: Glucose-dependent insulin release from genetically engineered K cells. *Science* 290(5498):1959-62 (2000).
10. Egawa K, Maegawa H, Shimizu S, Morino K, Nishio Y, Bryer-Ash M, **Cheung AT**, Kolls JK, Kikkawa R, Kashiwagi A: Protein-tyrosine phosphatase-1B negatively regulates insulin signaling in L6 myocytes and Fao hepatoma cells. *J Biol Chem* 30;276(13):10207-11 (2001).
11. Wang J, **Cheung AT**, Kolls JK, Starks WW, Martinez-Hernandez A, Dietzen D, Bryer-Ash M: Effects of adenovirus-mediated liver-selective overexpression of protein tyrosine phosphatase-1B on insulin sensitivity *in vivo*. *Diabetes Obes Metab* 3(5):367-80 (2001).
12. Huang D, **Cheung AT**, Parsons JT, Bryer-Ash M: Focal adhesion kinase (FAK) regulates insulin-stimulated glycogen synthesis in hepatocytes. *J Biol Chem* 17;277(20):18151-60 (2002).
13. Lam NT, Lewis JT, **Cheung AT**, Luk CT, Wang J, Bryer-Ash M, Kolls JK, Kieffer TJ: Leptin increases hepatic insulin sensitivity and protein tyrosine phosphatase-1B expression. Mol Endocrinol.18(6):1333-45 (2004).
14. Lam NT, **Cheung AT**, Cheeseman CI, Kieffer TJ: Leptin reduces glucose transport and cellular ATP levels in INS-1 β-cells. J Mol Endocrinol. 32(2):415-24 (2004).
15. **Cheung AT**, Lewis JT, Dayannandan B, Boylan MO, Wolfe M, Kieffer TJ: Meal- regulated insulin production from gut K-cells – putative targets for insulin gene transfer to treat type 1 diabetes. (Submitted).

Book Chapters

Cheung AT and Kieffer TJ: Gene Therapy for Metabolic Diseases. *Diabetes Mellitus: A Fundamental and Clinical Text* , 3rd Edition, LeRoith D, Taylor SI & Olefsky J, eds., Lippencott, Philadelphia, USA, 2003

Abstracts

1. Bhanots S, Bryer-Ash M, **Cheung A** , McNeil JH: Bis(maltolato)oxovanadium (IV) Lowers Plasma Insulin and Blood Pressure in Spontaneously Hypertensive Rats. *Proc Annu Meet Endocrine Society* 96:184B, (1993).
2. **Cheung AT**, Pederson RA, Bryer-Ash M: Feedback Regulation of Glucose-dependent Insulinotropic Polypeptide by Insulin. *Clinical Research* 42 (1): 20A, (1994).
3. **Cheung AT**, Pownall S, Jirik F, West E, Bryer-Ash M: Identification and cloning of a novel non-receptor type protein tyrosine phosphatase from human adipose tissue. *Program FASEB Annual Meeting*, New Orleans, LA. (1996).
4. **Cheung AT**, Kolls JK, Hodges N, Bryer-Ash M: Overexpression of TNF-α inhibitory protein reverses insulin resistance in obese Zucker rats: A novel method for studying mechanisms of TNF-α induced insulin resistance. *Diabetes* 46 (suppl 1):873A, (1997).
5. **Cheung AT**, Bryer-Ash M, Jansen D, Bandyopadhyay D, Wanstrath R, Kusari A and Kusari J: Protein tyrosine phosphatase -1B (PTP-1B) activity is markedly impaired despite increased PTP-1B levels in adipose tissue of obese humans. *Diabetes* 46 (suppl 1): 1081A, (1997).
6. Ree D, **Cheung AT**, Fuselier J, Kolls JK, Coy DH, Bryer-Ash M: Inhibition of TNF-α improves peripheral and hepatic insulin sensitivity in obese Zucker rats: Differential effects of TNF-α on insulin signaling. *Diabetes* 47 (suppl. 1):25A, (1998).
7. **Cheung AT**, Wang JF, Bryer-Ash M: A novel non-isotopic method for in vivo measurement of serine/threonine phosphorylation of insulin receptors. *Diabetes* 47 (suppl. 1):A331, (1998).
8. **Cheung AT**, Ree D, Kolls JK, Bryer-Ash M: Identification of Focal Adhesion Kinase as the point of cross-talk between insulin and tumor necrosis factor-α (TNF-α) signaling pathways in the liver. Presented at the Joslin Diabetes Center, Centennial Celebration, "From Genes to Patients and Back". Boston, MA. (1998).
9. Wang JF, **Cheung AT**, Kolls JK, Bryer-Ash M: The role of protein-tyrosine phosphatase-1B (PTP-1B) in the regulation of Focal Adhesion Kinase (FAK) by insulin. *Diabetes* 48 (suppl. 1):A328, (1999).
10. **Cheung AT**, JF Wang, Ree D, Kolls JK, Bryer-Ash M: Tumor necrosis factor-alpha (TNF-α) prevents insulin induced tyrosine phosphorylation of Focal Adhesion Kinase (FAK) in HepG2 cells via upregulaion of leukocyte antigen related tyrosine phosphatase (LAR) expression. *Diabetes* 49 (suppl. 1): A327 (2000).
11. **Cheung AT**, Wang JF, Ree D, Kolls JK, Bryer-Ash M: Tumor necrosis factor-alpha (TNF-α) induces hepatic insulin resistance in obese Zucker (*fa/fa*) rats via interaction of leukocyte antigen related tyrosine phosphatase (LAR) with Focal Adhesion Kinase (FAK). *Diabetes* 49 (suppl. 1): A327 (2000).

12. **Cheung AT**, Dayanandan B, Lewis JT, Korbutt GS, Rajotte RV, Boylan MO, Wolfe MM, Kieffer TJ: A novel gene therapy target for diabetes: expression of insulin in gut K-cells. American Diabetes Association 60th Scientific Sessions Presidential Poster Session, San Antonio, TX. (2000).
13. Lam NT, **Cheung AT**, Kieffer TJ: Leptin reduces glucose transport and GLUT2 phosphorylation in beta cells. CDA/CSEM Proceedings, A139 Halifax (2000).
14. **Cheung AT**, Lewis JT, Dayanandan B, Kieffer TJ: Meal-regulated insulin production from intestinal K-cells. *Diabetes* 50 (suppl 2):A6 (2001).
15. Huang D, **Cheung AT**, Parsons JT, Bryer-Ash M: Focal Adhesion Kinase (FAK) regulates insulin-stimulated glycogen synthesis in hepatocytes. *Diabetes* 50 (suppl 2):A293 (2001).
16. Wang JF, **Cheung AT**, Kolls JK, Starks WM, Martinez-Hernandez A, Dietzen D, Bryer-Ash M: Effects of adenovirus-mediated liver-specific overexpression of protein-tyrosine phosphatase-1B (PTP-1B) on insulin sensitivity *in vivo*. *Diabetes* 50 (suppl 2):A327 (2001).
17. Lam NT, Luk CT, Lewis JT, **Cheung AT**, Kieffer TJ: Leptin acutely increases hepatic insulin sensitivity and PTP-1B levels. *Diabetes* 50 (suppl 2):A369 (2001).
18. Egawa K, Maegawa H, Shimizu S, Morino K, Nishio Y, Bryer-Ash M, **Cheung AT**, Kolls JK, Kashiwagi A, Kikkawa R: Protein-tyrosine phosphatase-1B negatively regulates insulin signaling in L6 myocytes and Fao hepatoma cells. *Diabetes* 50 suppl 2:A401 (2001).

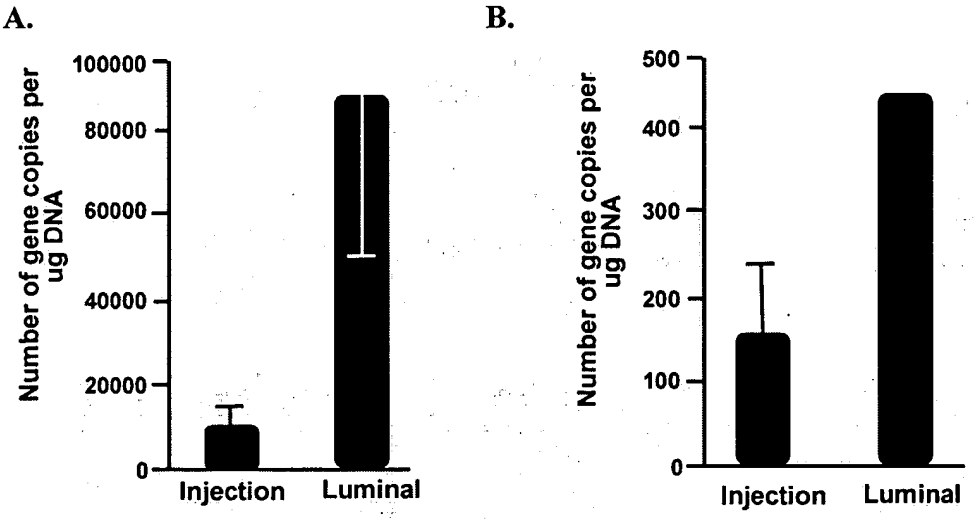

Figure 1: A. Numbers of GFP gene copies in duodenal mucosal tissue 2 days after AAV vector administration to mouse duodenum by direct injection and luminal incubation (n=3 for both groups). **B.** Numbers of GFP gene copies in duodenal mucosal tissue at 14 days after AAV vector administration to mouse duodenum by direct injection and luminal incubation (n=3 and 2 for injection and luminal incubation, respectively). Values shown are mean ± SEM.

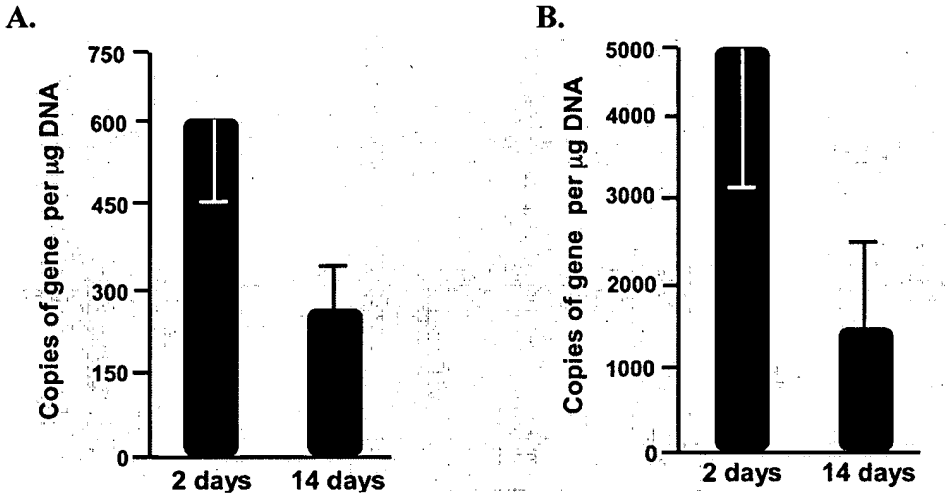

Figure 2: A. Numbers of DsRed gene copies in duodenal mucosal tissue at 2 and 14 days after FIV vector administration to mouse duodenum by direct injection (n=4 and 3 for 2 days and 14 days, respectively). **B.** Numbers of DsRed gene copies in duodenal mucosal tissue at 2 and 14 days after FIV vector administration to mouse duodenum by luminal incubation (n=3 for both groups). Values shown are mean ± SEM.

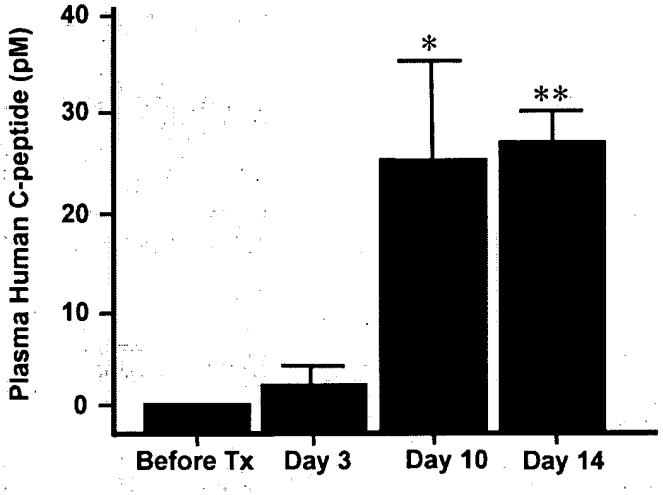

Figure 3: Plasma human C-peptide levels in FIV-cgA/hIns-treated rats. FIV virus vector solution was injected in the duodenum (n=10), and plasma samples collected and assayed for human C-peptide at days 3, 10 and 14. Data shown are mean ± SEM. One-tail paired student t-test was used to determine statistical significance. * $p<0.03$ and ** $p<0.0005$ vs. values before vector treatment.

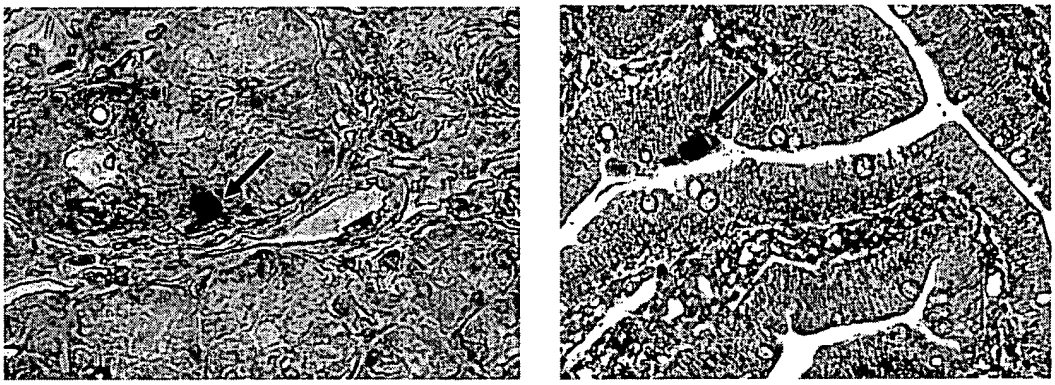

Figure 4: Immunohistochemical staining of human insulin in paraffin embedded sections of duodenum from FIV-cgA/hIns treated rat. Arrows indicate human insulin immunoreactive cells.